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Award Number: DAMD17-03-1-0020

TITLE: Early Detection of Prostate Cancer with a New Data Acquisition System for Telomerase Activity Analysis

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REPORT DATE: April 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2004	Annual (1 Apr 2003 - 31 Mar 2004)

4. TITLE AND SUBTITLE

Early Detection of Prostate Cancer with a New Data Acquisition System for Telomerase Activity Analysis

5. FUNDING NUMBERS

DAMD17-03-1-0020

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Prostate cancer is the most common malignancy that requires a screening of a very large population at risk. In addition, there is a crucial necessity not only to detect prostate cancer at potentially curable stage, but also to distinguish between the most aggressive and indolent carcinomas. At present time, there is a lack of satisfactory tools for early detection of prostate cancer. In this context, our research team that includes physicists, molecular biologists and urologists is working enthusiastically on the development of the instrument and assay for prostate cancer screening, and in a parallel, we are studying correlations between telomerase activity and clinicopathological manifestations of prostate cancer. During this period we have accomplished he following: a) characterized limits of sensitivity of the proposed technology that is at least 10-100 folds higher compared to standard TRAPeze XL assay; b)optimized sample collection, processing and electrophoresis; c) created a tissue bank for determination of a clinical applicability of the proposed instrument; d) obtained important preliminary data on clinical samples; e) reported current data on two AACR meetings; f) prepare two papers for submission; and g) submitted the proposal for clinical trial development award.

14. SUBJECT TERMS

Prostate Cancer, early detection, telomerase activity

15. NUMBER OF PAGES

17

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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ANNUAL REPORT

Early Detection of Prostate Cancer with a Novel Data Acquisition System for Telomerase Activity Analysis

INTRODUCTION

Since prostate cancer is the most common malignancy that affects about 30% of young men, and 64% of men 60 to 70 years of age (Sakr et al., 93; 94; Walsh, 94), it requires a screening of a very large population at risk. In addition, there is a crucial necessity not only to detect prostate cancer at potentially curable stage, but also to distinguish between the most aggressive carcinomas that carry the potential to develop into lethal disease and need more aggressive treatment, and indolent carcinomas that require monitoring. However, heterogeneous and multifocal character of the disease significantly complicates a collection of representative tumor samples subjected for standard clinical analysis, and at present time, there is a lack of satisfactory tools for early detection of prostate cancer. Therefore, there is a crucial need to develop noninvasive yet highly sensitive and specific assays for prostate cancer screening and monitoring. In this context, the proposed study is focused on the development of the diagnostic and prognostic tool for early noninvasive prostate cancer detection and monitoring of the disease progression. Our research team that includes physicists, molecular biologists and urologists is working enthusiastically and in a parallel on the development of the instrument and assay, and on the studying correlations between telomerase activity and clinicopathological manifestations of prostate cancer.

BODY

Approved Statement of Work

Task 1. **To evaluate the prototype of *SB Telomerase Analyzer* for quantitative measurement of telomerase activity, using different ratios of prostate cancer cells (LNCaP and/or PC-3) versus normal cells as a model of disease (Months 1-12):**

a) Characterize the sensitivity of the proposed technology in present configuration using different ratios of malignant cells versus normal cells (up to single cancer cell). Determine the dynamic range of our instrument.

b) Optimize the primer design, fluorescence labeling, PCR conditions etc. in order to avoid false positive results (specificity tests). Test identical aliquots of each sample with and without telomerase inactivation using direct detection and electrophoretic separation of PCR product.

c) Determine the reproducibility of the proposed method by consequent run of the same sample, under standard conditions.

Task 2. **Demonstration of the applicability and the advantages of the proposed data acquisition system for early diagnosis of prostate cancer, using quantitative measurement of telomerase activity in exfoliated cells in voided urine samples (Months 6-24).**

a) Comparative analysis of the telomerase activity measurements by the proposed data acquisition system with cytological analysis of exfoliated cells in voided urine specimens from prostate cancer patients and normal individuals.

b) Statistical analysis of the obtained results in relation to standard clinical tests.

Task 3. **Optimization of hardware, software and experimental protocols for clinical application (Months 1-24).**

a) **Further increase of the sensitivity of the proposed technology.**

b) **Redesign the capillary injection site for programmable operation.**

c) **Modification and optimization of software for specific telomerase data acquisition.**

d) **Final optimization of all protocols for clinical application.**

During the first year, we made a significant progress toward the proposed specific aims, and in particular, toward the determination of the sensitivity and reproducibility of the proposed prototype of the SB telomerase analyzer (task 1), potential applicability for clinical use (task 2), and optimization of all experimental protocols (task 3). Sensitivity tests that claim single cell resolution using serial dilutions of homogeneous cell line extracts in pure reagents and high number of PCR cycles to compensate limited sensitivity of standard methods of detection of PCR products, are not accurate for several reasons. Firstly, they do not reflect real clinical conditions, especially during early stages of cancer development when small population of malignant cells is surrounded by predominant population of other types of cells that can contain unfavorable factors not only for telomerase activity, but also for PCR efficiency. Secondly, PCR conditions at low and high degree of cell lysate dilution are incomparable. In addition, high number of PCR cycles ultimately leads to false-positive results.

In accordance with the approved Statement of Work, we started our project with optimization of all methodological steps including PCR conditions, fluorescent labeling and modifications of the detection modules. We have tested a sensitivity of the proposed instrument first using a conventional TRAPeze XL assay and end-point PCR (Perkin Elmer GenAmp PCR System 2400). Then we have continued these tests on the model of prostate cancer, different ratios of telomerase-positive prostate cancer PC-3 cells in normal cells surrounding, using a real-time PCR (Opticon MJ Research) for the amplification of telomerase activity products (RTQ-TRAP assay). Real-time PCR is more advanced compare to end-point PCR, allows quantitative comparative analysis of amplified products, and makes the testing of the proposed instrument more meaningful.

First of all, our data have confirmed that at present configuration, the proposed instrument is at least 10-100 times more sensitive in the detection of telomerase activity products compared to the analysis by commercial TRAPeze XL kit since we can reliably detect less than 0.00002 amole of TSR8 synthetic template (a minimal amount for TRAPeze XL is 0.00032 amole). To reach the maximum *detection sensitivity*, we investigated the use of single-photon detection techniques within the context of multicolor fluorescence detection methodology based on multicolor fluorescence excitation. We have found that appropriate combination of these techniques offers a dramatic increase in the sensitivity. The most important result of this study was the determination of the minimum number of photons required for a reliable identification of a fluorescent dye: the identified number was as low as ~100 per identified color, that is ~5 orders of magnitude less than fluorescence signals detected in commercial sequencing machines. Therefore, the intrinsic sensitivity of the technique can be traded off for additional advantages, including more convenient design and lower cost. In addition, SB instrument has much larger *dynamic range* in comparison with even such an advanced instruments as real-time PCR Opticon MJ Research since the proposed instrument can reliably detect fluorescence signals from as low as 0.00002 amole to as high as several million telomerase-positive cells. The proposed instrument's detection system is based on the single photon count and direct reading in digital form. A large dynamic range permits detection of both very high and very low signals. Because of the use of single photon counting, the dynamic range of the proposed instrument is determined by the maximum detected count rate and the noise associated with minimum detected

signal. Using our proprietary design for wide-band pulse amplifier, we already demonstrated measurement of a count rate as high as 10^7 *c/s;* the noise associated with the dark count of the PMT is about 20-30 *c/s*. Therefore, if we define a dynamic range as a ratio of maximum measured signal to minimum noise we obtain the dynamic range higher than 300,000 (compare to 6,000-7,000 dynamic range of best 16 bit CCD devices used in commercial DNA sequencing machines). *These features, higher sensitivity and larger dynamic range, are an absolute prerequisite for use in clinic where patient's samples can have unpredictable levels of telomerase activity, from very high to extremely low.*

Analysis of Telomerase activity by SB Telomerase Analyzer and Real-Time PCR

In order to determine limits of sensitivity of the proposed instrument, we have carried out telomerase activity tests on prostate cancer cell lines using SB DNA sequencer and standard TRAPeze XL kit (InterGen Inc., CA) with fluorescein-labeled reverse primer (485 nm excitation). After 32 PCR cycles, samples were diluted 1:10 in formamide, denatured at 96° C for 3 min, and further serially diluted in deionized water. The electrophoretic injection and separation regimes were 3.5 KV, 30 sec, and 15 KV, 15 min, respectively. For fluorescence excitation we used 488 nm Ar-ion laser.

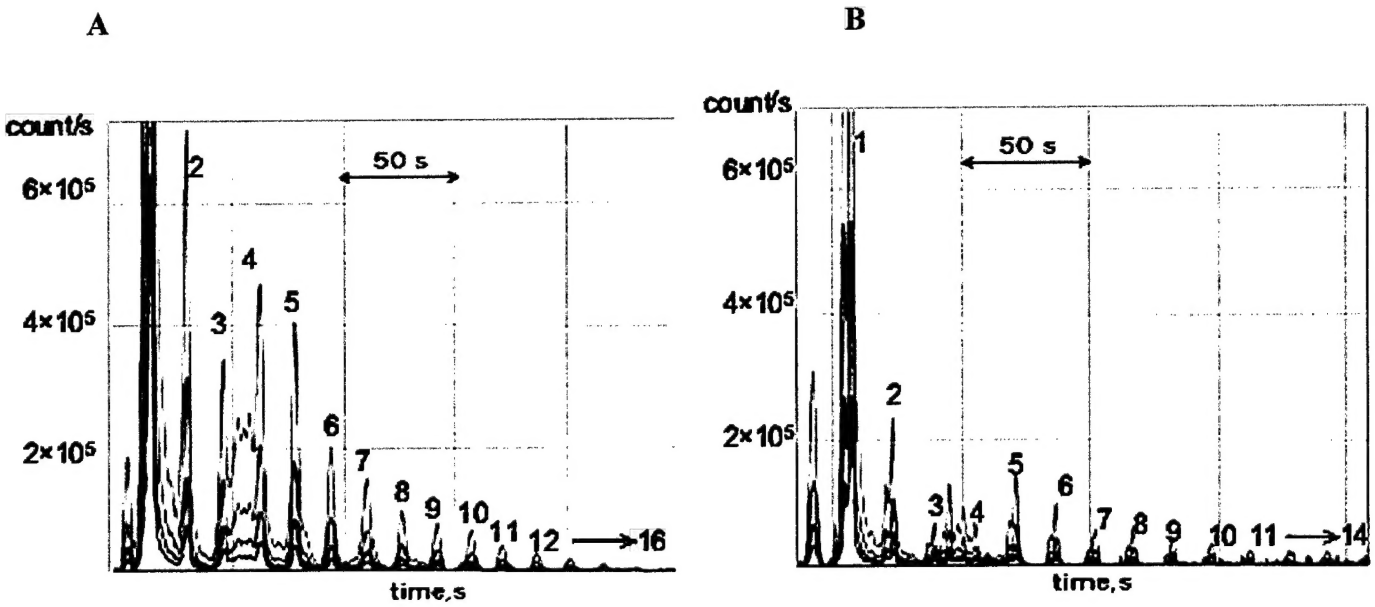

Figure 1. Analysis of telomerase activity in prostate cancer PC-3 cells by capillary electrophoresis.

The SB instruments demonstrated a reliable detection of telomerase activity product from as high as 100,000 cells (A) and as low as ~10 cells (B). We were able to detect at least 14-16 equidistant peaks corresponding to 6 base pairs telomeric repeats; the amplitude of the first peak was more than 6×10^6 counts per second. Total photon count for first 6 peaks integrated over all four spectral ranges was about 100,000,000 counts. (Four different colored lines represent four different filters in the detection system). In addition, this characteristic 6 base pair pattern is a "signature" of the telomerase activation.

Our data acquisition system allows multicolor, two-dimensional presentation of the PCR amplification of telomerase activity products. Multicolor fluorescence detection offers several

advantages. Firstly, it enables for simultaneous running a test sample and internal control in one capillary lane (Fig. 2). Secondly, it allows increase the instrument sensitivity due to application of correlation techniques for processing data with high noise level. In addition, the proposed way of data analysis is more accurate, easier and more reliable for interpretation than conventional approaches. Data processing is automated and takes only a few minutes.

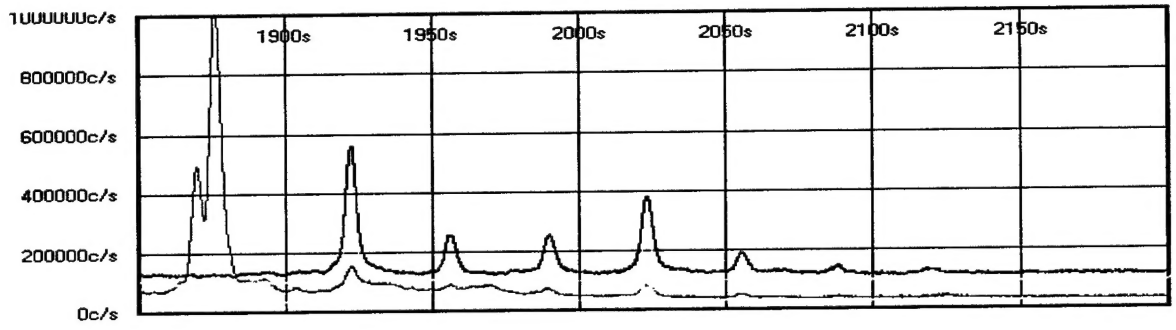

Figure 2. Analysis of PCR amplification products from 0.00016 amole of TSR8 template (synthetic oligonucleotide that contains 8 telomeric repeats).

One 532 nm laser allows detection of both fluorescence labeled internal control (red) and TSR8 (black). Even this extremely low amount of template (the minimal amount that was analyzed by TRAPexe XL kit is 0.0032 amole that is 20 folds higher), and not optimal laser wavelength, allow clear photon count (550,000 counts/sec) and very low noise (left).

Since the proposed instrument is able to detect the telomerase activity of ~10 cancer cells with very high integrated photon count (Fig. 1), it can detect telomerase activity with indeed single cell resolution. As a next step, currently, we are testing this statement on different ratios of telomerase-positive prostate cancer PC-3 cells in telomerase-negative surrounding using quantitative TRAP assay and real-time PCR amplification of telomerase activity products. An optimized RTQ-TRAP assay protocol for PCR amplification of telomerase activity products is as follow. A real-time PCR is carried out in a 96-well plate. The sequence of TS and ACX primers was not modified (TS: 5'-AATCCGTCGAGCAGAGTT-3'; ACX: 5'-GCGCGGCTTACCCTTACCCTTACCCTAACC-3'), however, instead of two fluorescent dyes conjugated with each primer, we used a standard SYBR Green protocol. A fluorescence dye SYBR Green is capable of binding with exceptionally high affinity to the double-stranded amplicons and generating fluorescence signals after each PCR cycle, allowing continuous, real-time measurement of PCR products that were analyzed by the Opticon software and with the proposed TA analyzer. Total reaction volume was 20 μl per well, containing 10 μl of 2 x SYBR Green Master Mix (Qiagen, CA), 0.1 μg of each primer, 2 μl of protein extract and 7.8 μl of RNase-free water. The reaction mixture was first incubated at 25° C for 20 min to allow the telomerase presented in the protein extract to elongate the TS primer by adding TTAGGG repeat sequences. The PCR was then started at 95° C for 15 min (hot start) to activate the Ampli*Taq* polymerase, followed by 40-cycle amplification: denaturation at 95° C for 20 sec; annealing at 55° C for 30 sec; extension at 72° C for 60 sec; plate reading at 60° C for 10 sec. All samples were run in duplicates. Serial dilutions of PC-3 cell extract were used in each real-time PCR run as a standard and positive control; reaction mixture with 2 μl of CHAPS lysis buffer instead of protein extract (no target) was used as a negative control.

At present time, we are working on the adaptation of the real-time PCR protocol for testing of

amplified telomerase activity products by SB instrument. Since now we use SYBR Green dye for fluorescent labeling during the real-time PCR, we need to make several modifications of the detection system, including laser type and filters, as well as modification of gel type for capillary electrophoresis. Our experiments have shown that previously used ABI's gels, POP-4 and POP-5, are not suitable for keeping telomeric fragments in double-stranded state, when SYBR Green can generate maximal fluorescence, therefore, we are testing now another types of ABI gels, such as Genescanpolymer, SSCP and others. PCR cycling was also modified in order to avoid the melting of amplified products after reading of fluorescence signals.

To determine limits of sensitivity of both PCR and SB instrument under conditions that more closely correspond to real prostate cancer pathology, characterized by low proportion of malignant cells in normal surrounding, we used different ratios of prostate cancer PC-3 cells in a surrounding of telomerase-negative cells. As a source of normal cells, we used exfoliated epithelial cells from urine of normal patients that did not express any telomerase activity, but presumably contained different kinds of inhibitors, including telomerase enzyme inhibitors and PCR inhibitors. Of interest, several tested cell lines, widely referenced as *telomerase negative*, including IMR 90, HS 27, and NIH 3T3, expressed detectable telomerase activity (see Fig. 7). These results were reported on Annual AACR meeting (Botchkina et al., 94). Therefore, the use of extracted (non cultured) cells that do not contribute to the detected telomerase activity, but contain different inhibitors is more accurate and meaningful for determination of the instruments sensitivity.

Our experiments have demonstrated that even in the absence of tissue inhibitors, when only homogenous telomerase-positive cell lines were used, the lowest detectable number was about 100 cancer cells (***Figure 3)***.

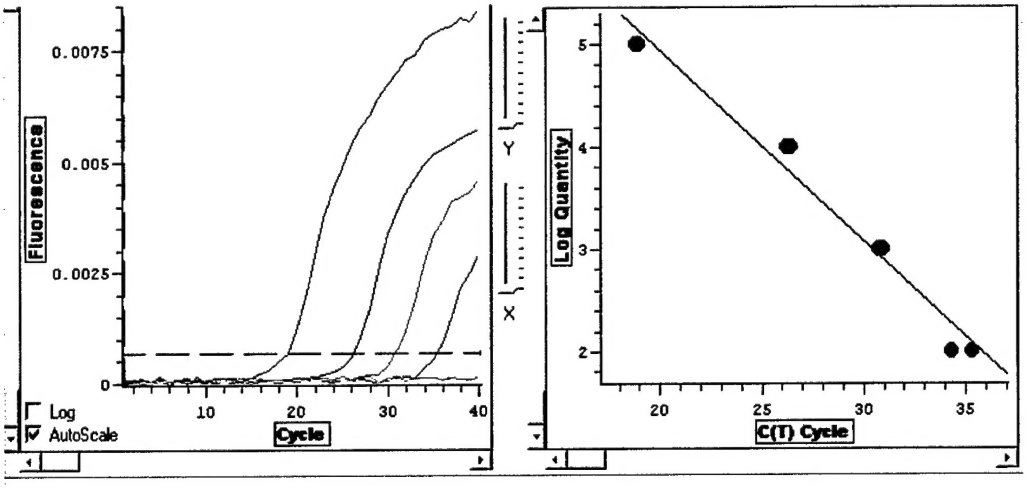

Figure 3 represents typical real-time PCR run for analysis of telomerase activity in serial dilutions of 100,000 PC-3 cells. Note that even under ideal conditions (pure telomerase-positive cancer cell line, pure PCR reagents, no tissue inhibitors), the limits of sensitivity of real-time PCR are about 100 cells.

As we expected, when extracts from exfoliated telomerase-negative cells from patients urine samples were added into reaction mixture, we observed a significant shift of the amplification curve that reflects an inhibition of telomerase activity and PCR efficiency by tissue factors present in patients samples (***Figure 4)***.

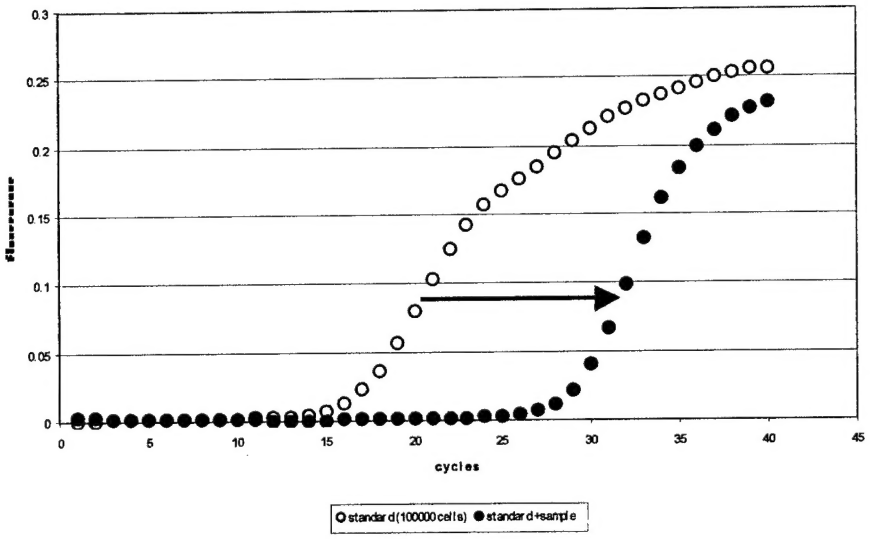

Figure 4. Inhibition of the PCR efficiency by the presence of tissue inhibitors.
PCR amplification of telomerase activity products from protein extract of 100,000 PC-3 cells (empty dots), and the same amount of protein extract, combined with the cellular extract from normal patient urine sample (black dots). Note the shift of the amplification curve after addition of the tissue extract from patients sample that contains inhibitors of telomerase activity and PCR inhibitors. Such a shift corresponds to the 1000 fold loss of the instrument sensitivity.

These observations are very important because 1) they show the necessity for more sensitive instruments with larger dynamic range for use in clinic; and 2) provide an additional argument for the necessity of the back up control for all samples with low or negative measurements of telomerase activity. This approach can decrease false-negative measurements, thereby increasing an assay's accuracy.

We tested the possibility of false-positive results (non-specific amplifications during PCR) by avoiding a target in the reaction mixture, or by increasing the degree of sample dilutions. Our experiments have demonstrated that under these standardized conditions, non-specific amplification does not occur. Linearity and accuracy of RTQ-TRAP assay and SB analyzer were tested using the same samples (serially diluted extracts of PC-3 cells) analyzed in separate experiments in duplicates. These tests demonstrated an accurate reproducibility of capillary electrophoresis and real-time PCR (all statistical analyses will be carried out in the current year). However, we need to minimize manual steps, such as timer-controlled sample injection, which should additionally improve an accuracy of measurements.

Tissue Bank

In accordance with *Task 2*, after obtaining of the HSRRB approval, we have started creation of a tissue bank for evaluation of the proposed assay in clinical conditions during the current year. By now, we collected 54 samples of exfoliated cells from naturally voided urine specimens of prostate cancer patients and normal individuals subjected to routine medical examination and prostate cancer screening. Analysis of telomerase activity in exfoliated cells in urine has a high potential as a

molecular marker for early detection of prostate cancer. First of all, in contrast to unpredictable features of heterogeneous surgically removed specimens, exfoliated cancer cells in body fluids fully represent corresponding primary tumors, so they can indicate not only the fact of tumor presence, but can also predict future tumor behavior, if suitable molecular markers were used.

Since telomerase is unstable ribonucleoprotein, we standardized and optimized the protocol for urine sample collection, processing and storage in order to protect telomerase enzyme from degradation. Briefly, fresh naturally voided 50-100 ml of urine (excluding the first morning specimens in which telomerase activity can be affected by the long time exposure in aggressive conditions) were collected in sterile plastic containers after prostatic massage. Immediately after collection, urine specimens were put on ice and centrifuged as soon as possible (not later than in 15-20 minutes) at 2500 rpm, +4° C, for 7 minutes. Pelleted cells were then washed in 10 ml of cold PBS (pH 7.4), and then in 2 ml of PBS containing 100 U/ml of RNase and protease inhibitor cocktail (SUPERase-In; Ambion, CA). Using our protocol, we were able to detected telomerase activity in all prostate cancer cases.

Analysis of Telomerase Activity in Clinical Samples (Preliminary Data)

Our preliminary results confirmed that telomerase activity can be detected in exfoliated cells from urine of prostate cancer patients, therefore our choice of the molecular marker for early detection of prostate cancer was right and promising. So far, the specificity of this test was 100% since we have detected telomerase activity in *all* prostate cancer patients (n=9). It is in agreement with growing data that telomerase has the highest sensitivity and specificity for cancer among more than one hundred suggested molecular makers. For comparison, the sensitivity of the urine cytology after prostate massage, even for patients with *advanced* prostate cancer is about 48%. Another important observation was that levels of telomerase activity in patient's samples usually are close to the limits of sensitivity even for such an advanced instrument as the Opticon MJ Research (its linear range is between 100,000 and about 50-100 cells). Therefore, it is obvious that further analysis by capillary electrophoresis, which is not only more sensitive, but also is more specific since it can demonstrate a "signature pattern" of telomerase activity (6 base pairs repeats; see Fig. 1), is highly necessary for clinical samples analysis in order to avoid false-negative and false-positive readings.

Since urine specimens can contain some other telomerase-competent cells (such as bacteria, blood cells, immune cells, fungi etc) that can affect detected telomeraze activity levels, we isolated all epithelial cells (wich also may or may not contain prostate cancer cells) from the rest of urinary cellular content by immunomagnetic cell separation (MACS; Miltenyi Biotec). Briefly, washed and pelleted exfoliated cells from urine specimens were resuspended in 400 µl ice-cold buffer and transfered to 2 ml microtube. One hundred microliters of HEA microbeads were added, mixed and incubated for 30 min at 4-6°C, followed by washing with 5 ml cold PBS containing 2mM EDTA, 0.5% BSA and 100 U/ml of SUPERase. Analyzed sample was placed into miniMACS separation column and *negatively* selected cells were discarded. Then, miniMACS column was removed from magnetic device, washed with 1ml PBS containing SUPERase and *positively* selected by immunomagnetic beads cells were also collected and protein extracts were prepared using CHAPS lysis buffer as described.

Under optimized real-time PCR conditions for Opticon MJ Research instrument, we set up standard curves for serial dilutions of telomerase-competent prostate cancer PC-3 cell extracts, equivalent to different cell count (from 1 mln cells to single cell). Figure 5 shows a representative real-time PCR run for telomerase activity analysis in patient with clinically confirmed prostate cancer.

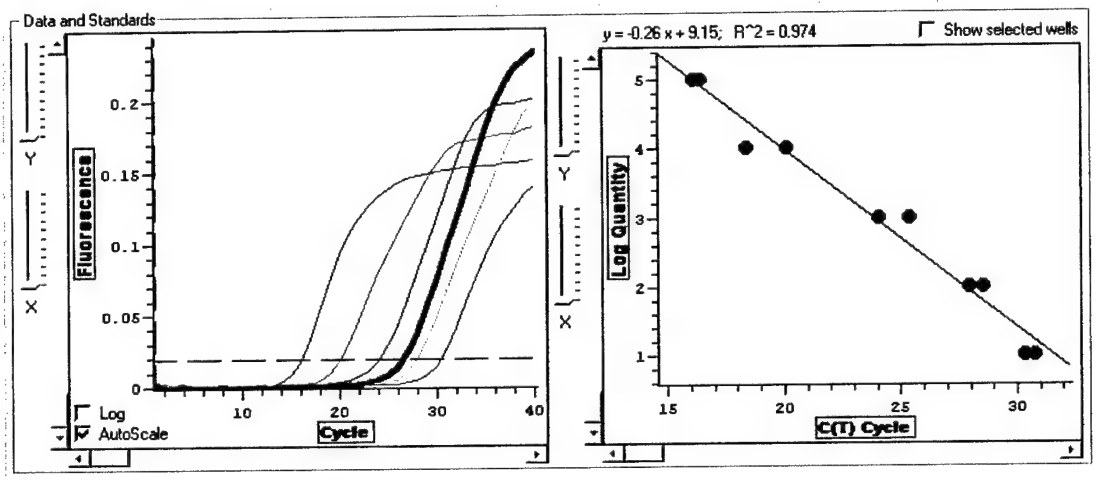

Figure 5. Telomerase activity in exfoliated cells from urine of patient with clinically confirmed advanced prostate cancer.
Real-time PCR amplification curves from protein extracts of 1:10 serially diluted 1million prostate cancer PC-3 cells (family of colored curves; left), and from urinary cellular content of patient with advanced prostate cancer (thick brown curve). Standard curve (right) for relative quantitation of telomerase activity levels.

However, the most interesting results we obtained during the screening of normal patients. Although all these patients were diagnosed with benign prostatic hyperplasia (BPH; n=41), ***no cancer*** was detected by standard clinical tests. In contrast, our data revealed three different subgroups on the basis of the teomerase activity, normalized to the number of PC-3 cells (Figure 6). First largest group did not show any amplification of telomeric repeates (53%) that most likely corresponds to the absence of malignant disease. A second group expressed relatively low levels of telomerase activity, equivalent to 1-91 PC-3 cells (37%). Third group demonstrated high relative telomerase activity, equivalent to 168-670 PC-3 cells (10%) that probably reflects the presence of prostate carcinomas that were not detected by current screening modalities.

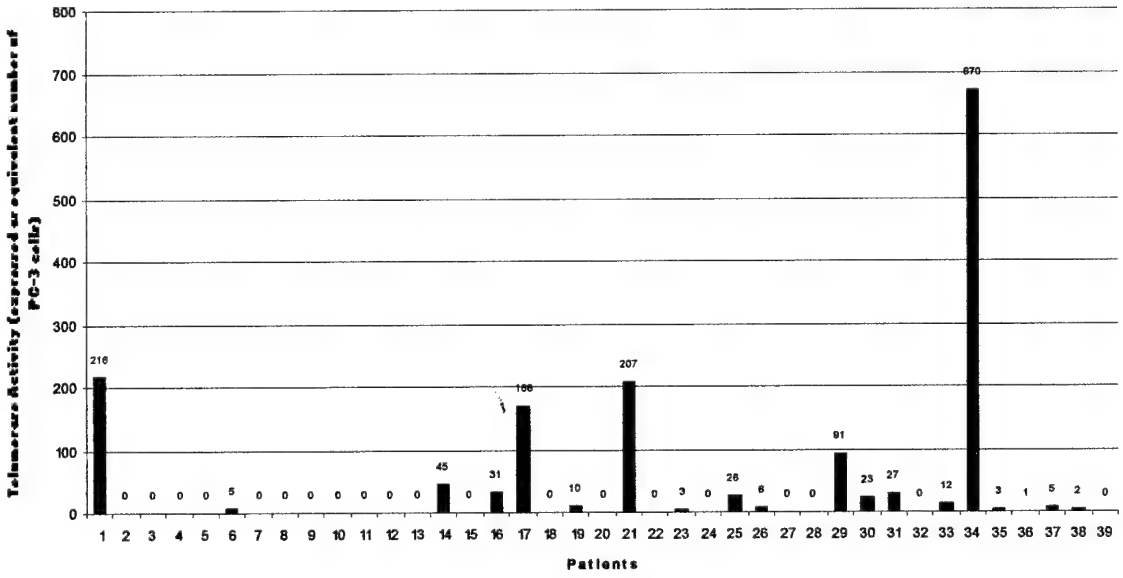

Figure 6. Different levels of telomerase activity in exfoliated cells from urine of patients with BPH.

Our data on comparative analysis of telomerase activity in cells with different invasive potential have demonstrated highest levels of activity in cells with highest metastatic potential, such as PC3-P and PC3-MM2 cells, compared to commonly used prostate cancer PC-3 cells (Botchkina et al., 04).

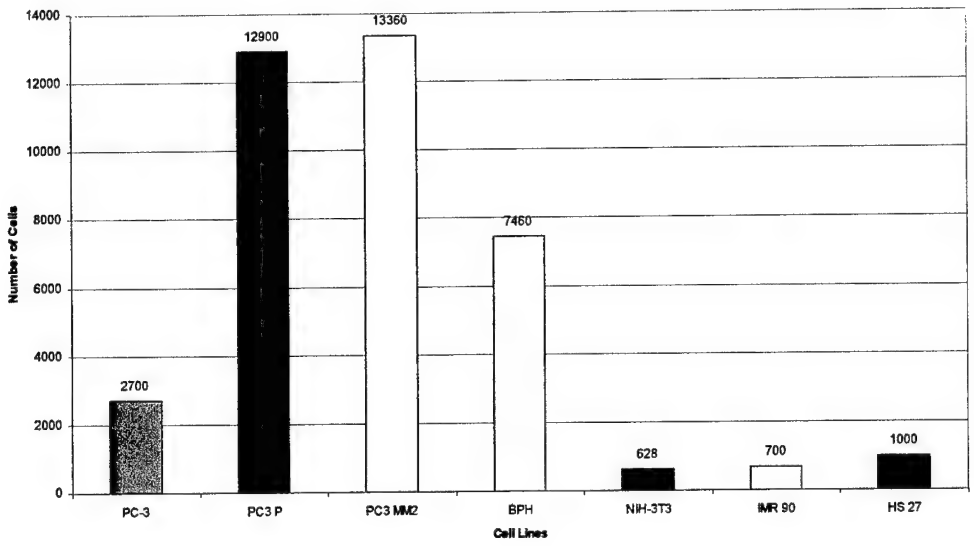

Figure 6. Levels of telomerase activity in cell lines with different invasive potential.

As it was noted above, quantitative analysis of telomerase activity in several cell lines that previously were referenced as telomerase-negative (such as HS 27, IMR 90 and NIH 3T3) revealed relatively low but detectable levels of activity.

Current lack of reliable diagnostic and prognostic criteria to distinguish between slowly developing and aggressive carcinomas leads to misconceptions and incorrect strategies of patient treatment. We hypothesize that differential levels of telomerae activity in a cohort of patients with BPH (no clinically confirmed prostate cancer) can be used for selection of potentially aggressive carcinomas that currently have no clinical manifestation. It is important that within this cohort of patients, none of the standard tests were sensitive enough to confirm prostate cancer or premalignant disease. Since this cohort would therefore contribute to new subcategory of prostate cancer cases, *it is extremely important to validate these new findings on a large population of men.* Therefore, as a logical continuation of this project, we suggest a development of a clinical trial on the validation of the proposed assay, based on quantitative analysis of telomerase activity by real-time PCR and capillary electrophoresis by SB telomerase analyzer, as a diagnostic and prognostic tool which can allow not only early detection of prostate cancer, but also selection of potentially aggressive carcinomas.

To provide a foundation for successful execution of our major goal, demonstration of the applicability and the advantages of the proposed data acquisition system for early diagnosis of prostate cancer, during the current year we will extend our tumor bank and carry out analysis of telomerase activity in clinical samples on SB Telomerase analyzer. Careful statistical analysis of correlations between telomerase activity and multiple clinicopathological data will allow us make a conclusion about clinical utility and applicability of the proposed instrument and assay as a diagnostic and prognostic marker for prostate cancer. The proposed assay is *noninvasive*, so it not only presents *no potential risks,* and requires *no additional efforts or medical cost*, but it also allows multiple repeated tests that can have a major impact on early prostate cancer screening and monitoring, improving the sensitivity and specificity of testing.

KEY RESEARCH ACCOMPLISHMENTS

1. We have characterized limits of sensitivity of the proposed technology using prostate cancer cell lines, and demonstrated at least 10-100 folds higher sensitivity compared to commercial TRAPeze XL assay.
2. We have determined limits of sensitivity of real-time PCR and started comparative analysis with capillary electrophoresis.
3. We have optimized sample collection, processing and storage that allowed us reliably measure telomerase activity.
4. We have optimized all experimental protocols for real-time PCR and capillary electrophoresis.
5. We created a tissue bank in order to carry out tests for clinical applicability of the proposed instrument as a tool for early prostate cancer detection.
6. Our preliminary data have demonstrated that telomerase activity was expressed in all prostate cancer patients, showing 100% sensitivity.
7. Our data on comparative analysis of telomerase activity in cells with different invasive potential have demonstrated that cells with more aggressive status have much higher telomerase activity.
8. We have discovered that the cohort of patients with benign prostatic hyperplasia (no cancer was detected by standard screening modalities) falls into three subcategory on the basis of telomerase activity levels that suggests that it may be used for discrimination between indolent and aggressive carcinomas.

REPORTABLE OUTCOMES

1. Current data were reported on AACR/Japanese Cancer Association meeting "Advances in Cancer Research", January 2004, and Annual AACR meeting, March, 2204.
2. Two abstracts were published in corresponding Proceedings of the AACR.
3. Two papers are preparing for submission to Cancer Research and Molecular Cancer Research journals by June, 2004.
4. A tissue bank was created (exfoliated cells after prostate massage from urine of cancer and normal patients, undergoing routine medical examination or cancer screening).
5. Since current standard screening modalities have insufficient sensitivity and specificity for early detection of the prostate cancer and discrimination between indolent and aggressive carcinomas, we were strongly motivated to submit our proposal for Clinical Trial Development Award (DOD, PCRP 04), as a logical continuation of the current project. This trial can facilitate a validation of the proposed assay and instrument as a tool for prostate cancer screening and monitoring.

CONCLUSIONS

During the first year of the project we demonstrated that the proposed assay has a high potential as a tool for early prostate cancer detection. First of all, the choice of the molecular marker of cancer, telomerase activity, was correct since it demonstrates so far 100% sensitivity for cancer. The sensitivity and large dynamic range of the proposed instrument allow reliable detection of telomerase activity in clinical samples with unpredictable number of tumor cells. The proposed assay is *noninvasive*, so it presents *no potential risks,* and requires *no additional efforts or medical cost*, so it allows repeated testing and monitoring of the disease progression. Our preliminary data on clinical

samples from prostate cancer and normal patients are very encouraging and require timely validation on a large patient population.

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APPENDIX

95th AACR Annual Me
March 27-31, 2004
Orange County Convention
Orlando, Florida

994 ▮Telomerase▶ activity analysis in human cell lines usinga real-time PCR (RTQ-TRAP) assay.

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◀**Telomerase**▶ has a high potential as a biomarker of ◀**cancer**▶, since its activity was demonstrated in about 90% of human cancers and all immortal cells, and in contrast, it is absent from most normal somatic tissues after birth. However, ◀**telomerase**▶ is activited not only during tumorigenesis *in vivo* and immortalization *in vitro*, but also in germ line, stem cells and some normal somatic cells with high proliferative potential. Although levels of ◀**telomerase**▶ activity (TA) in these cases were considered as low or undetectable by standard TRAP assays, the appearance of highly sensitive quantitative methods of detection, such as a real-time PCR, requires quantitative re-examination of ◀**telomerase**▶ status in human cell lines that are commonly used as a reference controls. Also, conflicting results were reported regarding cell cycle-dependent regulation of TA in normal and immortal cell lines. *Materials and Methods.* We used telomerase-positive ◀**prostate**▶ ◀**cancer**▶, PC-3 cells line, and commonly referenced as telomerase-negative, IMR90 and HS27 cells (all cells were obtained from the SUNY Cell culture/hybridoma facility). PC-3 cells were cultured in F12K medium; IMR90 and HS27 cells were cultured in DMEM, supplemened with 10% fetal calf serum. All cells were cultured at 37° C under 5% CO_2 in a humidified chamber. Quantitative real-time TRAP assay was carried out in a 96-well plate using the Opticon MJ Research instrument and optimized standard SYBR Green protocol for measurements of TA. For growth arrest in G2/M phase, subconfluent and confluent cells were treated with 0.5 μg/ml or 5 μg/ml of Nocodazole (Sigma) for 24 hours. For serum starvation of cells, a medium was supplemented with 0.5% FCS. For confluency experiments, cells were allowed to grow with one medium change after 5 days of culturing in specified medium supplemented with 10% FCS till full confluency, and after two weeks of growing, cells were treated with nocodazole as described. *Results.* Amplification plots revealed a cell number-dependent amplification of telomeric repeates on serial dilutions of all examined cell lines. Any nonspecific amplification was not occured since no difference in fluorescence signals in no template controls and at high degrees of sample dilution was detected. Highest ◀**telomerase**▶ activity was detected in PC-3 cells; both IMR90 and HS27 showed ~10-20 times lower TA. For PC-3 cells, insignificant decrease in TA was seen after confluency was reached. Treatment with nocodazole further reduced TA in both subconfluent and confluent cells. In contrast, confluent IMR90 and

HS27 cells had significantly lower TA compared to subconfluent ones. Nocodazole treatment reduced TA in subconfluent HS27 and IMR90 cells, and had no significant effect on TA levels in confluent cells.

Citation information: Proceedings of the AACR, Volume 45, March 2004.